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**(54) OSTEOGENIC GROWTH OLIGOPEPTIDES AND PHARMACEUTICAL COMPOSITIONS
CONTAINING THEM**

OSTEOGENE WACHSTUMS-OLIGOPEPTIDE UND DIESE ENTHALTENDE PHARMAZEUTISCHE
ZUSAMMENSETZUNGEN

OLIGOPEPTIDES DE CROISSANCE OSTEOGENE ET COMPOSITIONS PHARMACEUTIQUES
LES CONTENANT

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- **EMBO JOURNAL** vol. 11, no. 5, May 1992, EYNSHAM, OXFORD GB pages 1867 - 1873 I. BAB ET AL. 'Histone H4-related osteogenic growth peptide (OGP): a novel circulating stimulator of osteoblastic activity'
- **CHEMICAL ABSTRACTS**, vol. 110, no. 21, 22 May 1989, Columbus, Ohio, US; abstract no. 186143, E.P. KHARCHENKO ET AL. 'Highly active analogs of a plate-like peptides historphin and kapporphine' page 96; column L; & VOPR. MED. KHIM. vol. 35, no. 2, 1989 pages 106 - 109 cited in the application
- **BIOCHIMICA ET BIOPHYSICA ACTA** vol. 1178, no. 3, 13 October 1993 pages 273 - 280 Z. GREENBERG ET AL. 'Mitogenic action of osteogenic growth peptide (OGP): role of amino and carboxy-terminal regions in charge'
- 'Preparation and Bioassay of Bone Morphogenic Protein and Polypeptide Fragments', by M.R. Urist et al., In **Methods in Enzymology** Vol. 146 ed. by D. Barnes and D.A. Sirbasku, pub. Academic Press, 1987 pages 294-312

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EP 0 687 270 B1

Description

Field of the Invention

- 5 [0001] The invention relates to osteogenic growth oligopeptides which possess stimulatory activity on osteoblastic and fibroblastic cells.

Background of the Invention

- 10 [0002] It has been established that regenerating bone marrow induces an osteogenic response in distant skeletal sites and that this activity is mediated by factors released into the circulation by the healing tissue [Bab I. *et al* (1985) Calcif. Tissue Int. 37:551; Foldes, J. *et al* (1989) J. Bone Min. Res. 4:643; Einhorn, T.A. *et al* (1990) J. Bone Joint Surg. Am. 72:1374; Gazit D., *et al* (1990) Endocrinology 126:2607; Mueller, M. *et al* (1991) J. Bone Min. Res. 6:401]. One of these factors, a 14-amino acid osteogenic growth polypeptide (OGP), identical with the C-terminus of histone H4, has been recently identified [Bab, I. *et al* (1992) EMBO J. 11:1867; European Patent Applications Nos. 89201608.0 and 90301862.0]. A histone H4 fragment of the formula Tyr-Gly-Phe-Gly-Gly is disclosed in Kharchenko, E.P. *et al* (1989) Vopr. Med. Khim. 35(2):106-9 and in Kharchenko, E.P. *et al* (1987) Biull. Eksp. Biol. Med. 103(4):418-20. This peptide demonstrated analgesic and opioid activity.

- [0003] Synthetic 14-mer osteogenic growth polypeptide (sOGP), identical in structure with the native molecule, has been shown to be a potent stimulator of proliferation of osteoblastic and fibroblastic cells *in vitro*. This synthetic polypeptide also stimulates osteoblastic cell alkaline phosphatase activity. When injected *in vivo* to rats, at very small doses, the synthetic osteogenic growth polypeptide increases bone formation and trabecular bone mass.

- [0004] As in the case of other polypeptide growth regulators, such as growth hormone and insulin-like growth factor [Hintz, R.L. (1990) Horm. Res. 33:105], osteogenic growth polypeptide-binding protein/s (OGPBP) may protect the osteogenic growth polypeptide against proteolytic degradation [Bab, I. *et al* (1992) EMBO J. 11:1867].

- [0005] C-terminal modified analogues of the osteogenic growth polypeptide, such as [Cys¹⁵(NEM)]OGP-NH₂, bind to the OGPBP. The modified analogues do not share the OGP stimulation of cell proliferation and do not react with certain anti-OGP antibodies. These polypeptide analogues can be used to release OGP from its complex with an OGPBP. If the competitive reaction takes place in a tissue culture medium, previously incubated with cells or a biological fluid having peptidase activity, the released OGP becomes exposed to proteolytic degradation resulting from said peptidase activity. Nevertheless, the possibility existed that short peptides resulting from the proteolytic degradation may retain the OGP activity.

- [0006] Since the OGP molecule is too large for effective oral administration, it would be of therapeutic importance to find oligopeptides of six or less amino acids residues, that retain the OGP biological activity, such short oligopeptides could be modified into a stable pharmaceutical preparation suitable for oral or other systemic treatment of several pathological conditions, particularly conditions involving loss of bone tissue. In addition, the identification of such oligopeptides would be an essential step towards the definition of the minimal amino acid sequence that still retains the OGP activity, which may provide the basis for further drug design.

- [0007] The present invention indeed relates to such native or synthetic osteogenically active oligopeptides.

- 40 [0008] The following abbreviations are used herein:

OGP(s) - osteogenic growth polypeptide(s).

OGPBP(s) - osteogenic growth polypeptide binding protein(s).

irOGP - immunoreactive OGP.

- 45 sOGP - synthetic OGP.

Summary of the Invention

- [0009] The invention provides a biochemically pure oligopeptide having stimulatory activity on osteoblastic and/or fibroblastic cells, a molecular weight of from 200 to 1,000, and a formula selected from (a) Tyr-Gly-Phe-His-Gly; (b) Tyr-Gly-Phe-Gly; (c) Ac-Met-Tyr-Gly-Phe-Gly-Gly; and (d) Gly-Phe-Gly-Gly-NH₂.

- [0010] The invention also provides a method of isolating a biochemically pure oligopeptide defined as (a) above or having the formula (e) Tyr-Gly-Phe-Gly-Gly or (f) Gly-Phe-Gly-Gly from a biological sample comprising the steps of: (a) discarding from said biological sample peptides having a molecular weight lower than 3000; (b) incubating the medium obtained in step (a) with a polypeptide that binds to an osteogenic growth polypeptide binding protein/s and does not bind to an antibody directed against osteogenic growth polypeptide in the presence of protease inhibitors to compete said osteogenic growth polypeptide out from its complex with said osteogenic growth polypeptide binding protein/s; and (c) separating the immunoreactive osteogenic growth peptide from the reaction medium obtained in step (b) by chro-

matographic methods and to biochemically pure peptides having stimulatory activity on osteoblastic or fibroblastic cells prepared by this method.

[0011] In addition, the invention relates to pharmaceutical compositions for stimulating the formation of osteoblastic or fibroblastic cells, and consequent bone formation, comprising a therapeutically effective amount of oligopeptide (a), (b), (c), (d) or (f) according to the invention and a pharmaceutically acceptable carrier.

Brief Description of the Figures

[0012]

Figure 1 shows production of steady-state and total iOGP by osteoblastic ROS 17/2.8 and osteoblastic MC3T3 E1 cells and NIH 3T3 fibroblastic cells. During the measurement period the cells were grown in chemically defined medium containing 4% Bovine Serum Albumin (BSA). Medium aliquotes for iOGP determination were obtained immediately after the addition of the BSA-containing medium ("0" time-point) and at the indicated times thereafter. Steady state and total iOGP were determined as before [Bab *et al* (1992) EMBO J. 11: 1867].

Figure 2 is flow chart describing the purification of iOGP from 24h tissue culture medium prepared as described in Fig. 1 above using MC3T3 E1 cells. Peptides of a molecular weight lower than 3000 were separated by three repeated dilution/centrifugation cycles. The retentate was then incubated with [Cys¹⁵(NEM)]OGP-NH₂ and recentrifuged. The filtrate was collected, and the iOGP separated from the [Cys¹⁵(NEM)]OGP-NH₂ using reverse phase HPLC and further purified as shown in Fig. 3. The purified iOGP in the peaks seen in Fig. 3 were subjected to amino acid sequencing by automated Edman's degradation and tested for proliferative activity as shown in Fig. 4.

Figure 3 shows HPLC separation of A - iOGP and [Cys¹⁵(NEM)]OGP-NH₂ using reverse phase Vydac C4 column; and B - the two iOGP peaks, using Merck C18 reverse-phase column. Dashed and continuous lines represent light absorbance and immunoreactivity, respectively.

Figure 4 shows the effect of OGP(10-14) (A) and OGP(10-14)His¹³ (B) (see table D) (recovered from the respective B-1 and B-2 peaks of Fig. 3) on osteoblastic MC3T3 E1 cell number *in vitro*. Dashed line - effect of positive SOGP control. Data are mean \pm SEM of triplicate cultures.

Figure 5 shows the effect of synthetic OGP(10-14) (○—○—○—○—○) and OGP (10-14)His¹³ (▲—▲—▲) on osteoblastic MC3T3 E1 (A) and fibroblastic NIH 3T3 (B) cell number *in vitro*. Cell cultures were set and challenged as in Figs. 1 and 4. Cf. effect of positive sOGP control (□—□—□). Data are mean \pm SEM of triplicate cultures presented as the ratio of treatment to BSA only control (T/C ratio).

Figure 6 shows the effect of synthetic Ac-Met⁰OGP(10-14) on osteoblastic MC3T3 E1 (A) and fibroblastic NIH 3T3 (B) cell number *in vitro*. Cell cultures were set and challenged as in Figs. 1 and 4. Dashed line - effect of positive SOGP control. Data are mean \pm SEM of triplicate cultures presented as the ratio of treatment to BSA only control (T/C ratio).

Detailed Description of the Invention

[0013] OGP is a 14-residue polypeptide identified from regenerating bone marrow which has been shown to stimulate the proliferation and alkaline phosphatase activity of osteoblastic and fibroblastic cells *in vitro* and to increase bone formation and trabecular bone mass in rats when injected *in vivo*. The amino acid sequence of OGP is as follows:

[0014] Ala-Leu-Lys-Arg-Gln-Gly-Arg-Thr-Leu-Tyr-Gly-Phe-Gly-Gly Synthetic OGP, with an identical amino acid sequence and biological activity has been prepared by standard solid phase methodology. It has also been found that in different biological fluids OGP forms a complex with OGPBP(s) and that sOGP analogues modified at their C-terminal region can be used to competitively release the total iOGP from OGP-OGPBP complexes, as described above.

[0015] The inventors have found that if the competitive reaction takes place in a tissue culture medium, previously incubated with cells or a biological fluid having peptidase activity, the OGP released from the OGP-OGPBP complex becomes exposed to proteolytic degradation by said peptidase activity. Surprisingly, the inventors have found that short oligopeptides, which were found to be present in said competitive reaction medium, retain the stimulatory activity on osteoblastic and fibroblastic cells, and consequently on bone formation.

[0016] The invention thus provides a biochemically pure oligopeptide having stimulatory activity on osteoblastic and/or fibroblastic cells, a molecular weight of from 200 to 1,000, and a formula selected from (a) Tyr-Gly-Phe-His-Gly; (b) Tyr-Gly-Phe-Gly; (c) Ac-Met-Tyr-Gly-Phe-Gly-Gly; and (d) Gly-Phe-Gly-Gly-NH₂.

[0017] The invention also provides a method of isolating a biochemically pure oligopeptide having formula (a) or (e) of the invention and having stimulatory activity on osteoblastic or fibroblastic cells from a biological sample comprising the steps of: (a) discarding from said biological sample peptides having a molecular weight lower than 3000; (b) incubating the medium obtained in step (a) with a polypeptide that binds to an osteogenic growth polypeptide binding protein/s and does not bind to an antibody directed against osteogenic growth polypeptide in the presence of protease

inhibitors to compete said osteogenic growth polypeptide out from its complex with said osteogenic growth polypeptide binding protein/s; and (c) separating the immunoreactive osteogenic growth peptide from the reaction medium obtained in step (b) by chromatographic methods.

[0018] The peptides having molecular weights lower than 3000 can be discarded by, for example, ultrafiltration with a cut-off of 3000 MW. The protease inhibitors can be commercially available inhibitors, for example E-64, Leupeptin or PMSF or mixtures thereof. The separation of the immunoreactive osteogenic growth peptide in step (c) can be accomplished by available HPLC techniques. A specific embodiment of the method of the invention is described in the Examples.

[0019] As will be shown in the following Examples, the oligopeptides according to the invention, whether obtained by said method of isolation or synthesized, possess stimulatory activity on osteoblastic and fibroblastic cells, and may thus be of great therapeutical value. The invention therefore also relates to pharmaceutical compositions for stimulating the formation of osteoblastic or fibroblastic cells comprising a therapeutically effective amount of a biochemically pure oligopeptide having formula (a), (b), (c), (d) or (f) of the invention.

[0020] Preferred pharmaceutical compositions according to the invention comprise a therapeutically effective amount of an oligopeptide comprising the amino acid sequence Tyr-Gly-Phe-His-Gly or Gly-Phe-Gly-Gly or a mixture thereof; and a pharmaceutically acceptable carrier.

[0021] The pharmaceutical compositions of the invention may be particularly useful for the stimulation of osteoblastic cells and/or fibroblastic cells, and consequently enhanced bone formation in various pathological conditions, for example osteoporosis (or osteopenia of any etiology), fracture repair, healing of wounds, intraosseous implants and bone supplementation, or other conditions requiring enhanced bone formation.

[0022] Accordingly, the invention provides a biochemically pure oligopeptide of formula (a), (b), (c), (d) or (f) of the invention for use in a method of treatment of the human or animal body by surgery or therapy or in diagnosis, preferably wherein the method of treatment is for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, and other conditions requiring enhanced bone formation. The invention also provides the use of such peptides in the manufacture of medicaments for the use in such treatments.

[0023] The invention also provides the use of an oligopeptide having the formula (a), (b), (c), (d), (e) or (f) of the invention in the manufacture of a medicament for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation and other conditions requiring enhanced bone formation cells.

[0024] The magnitude of a therapeutic dose of a polypeptide of the invention will of course vary with the group of patients (age, sex, etc.), the nature of the condition to be treated and with the particular polypeptide employed and its route of administration. In any case the therapeutic dose will be determined by the attending physician.

[0025] Any suitable route of administration may be employed for providing a mammal, especially a human, with an effective dosage of a polypeptide of this invention. Intravenous and oral administration may be preferred.

[0026] The pharmaceutical compositions of the invention can be prepared in dosage units forms. The dosage forms may also include sustained release devices. The compositions may be prepared by any of the methods well-known in the art of pharmacy.

[0027] The pharmaceutical compositions of the invention comprise as an active ingredient an oligopeptide of formula (a), (b), (c), (d) or (f) of this invention or a mixture of such oligopeptides in a pharmaceutically acceptable carrier, excipient or stabilizer, and optionally other therapeutic constituents. Acceptable carriers, excipients or stabilizers are non-toxic to recipients at the dosages and concentrations employed, and include buffers, such as phosphate buffered saline and like physiologically acceptable buffers, and more generally all suitable carriers, excipients and stabilizers known in the art.

EXAMPLES

Example 1 - Purification and characterization of pentapeptides from tissue culture medium

Materials

[0028] Tissue culture ingredients were purchased from Biological Industries (Beit Haemek, Israel). Culture dishes were from Nunc (Roskilde, Denmark). BSA protease inhibitors and N-ethylmaleimide (NEM) were from Sigma Chemical Co. (St. Louis, MO; Cat No. A 7030). Centricon-3 microconcentrators were purchased from Amicon, Inc. (Beverly, MA). t-Boc-Gly OCH₂-Pam resin, N-Boc protected amino acid derivatives, N,N-dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), N,N-dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Applied Biosystems Inc. (Foster City, CA). Hydrogen fluoride (HF) was purchased from Matheson (Secaucus, NJ), Boc-3-L-Tyr(BZ1)-OH from Bachem (Torrance CA), p-Cresol from Aldrich

Chemical Co. (Milwaukee, WI) and Sephadex G15F from Pharmacia (Uppsala, Sweden). C18 reverse-phase column and acetonitrile were from E. Merck, Darmstadt, Germany. C4 reverse-phase column was from The Separation Group, Hesperia CA.

5 Methods

Measuring iROGP accumulation in tissue culture media

[0029] ROS 17/2.8 or MC3T3 E1 osteoblastic cells or NIH 3T3 fibroblasts were maintained in α -Minimal Essential Medium supplemented with 10% fetal calf serum (ECS) and subcultured twice a week. Cells for experiments were derived from maintenance cultures at confluency. For the experiment the cells were seeded in 25 cm² tissue culture flasks at 1×10^4 cells/cm². The cultures were incubated at 37°C in CO₂ air. For the initial 46 h the medium was supplemented with 10% FCS and 0.2% nucleosides/ribonucleosides followed by an additional 2 h starvation period under serum free conditions. Then the serum free medium was replaced by 8 ml medium containing 4% BSA. Half millilitre medium aliquots for iROGP determination were obtained immediately after the addition of BSA containing medium ("0" time-point) and 12, 24, 36 and 48 hrs thereafter. Steady state and total iROGP were determined in these aliquots as before [Bab *et al.* (1992) EMBO J. 11:1867]

Separation between iROGP and OGPBP

[0030] Fig. 2 is a schematic demonstration of the separation between the iROGP and the OGPBP. A 3.75 ml sample of medium collected 24 h after cell starvation was diluted (1:1) with an equal amount phosphate buffered saline (PBS). The diluted medium was centrifuged in multiple centricon-3 microconcentrators for 1.5 h at 5,000 x g. Polypeptides smaller than 3000 MW were washed out of the retentate by three repeated dilution/centrifugation cycles using 1 mM sodium azide in 165 mM ammonium acetate pH 7.0 as diluent. The minimal retentate volume allowed in each micro-concentrator was 250 μ l. To release iROGP from the OGP-OGPBP complex, the 1:1 diluted retentate was incubated for 30 min at 37°C with 450 nmol/ml [Cys¹⁵(NEM)]OGP-NH₂ in 165 mM ammonium acetate containing 50 μ M E-64, 50 μ M Leupeptin and 500 μ M PMSF and recentrifuged.

30 Separation between iROGP and [Cys¹⁵(NEM)]OGP-NH₂

[0031] The filtrate obtained by the microconcentration step was partially evaporated to final volume of 600 μ l. The iROGP content of the filtrate was 0.31 nmol. The filtrate was divided into three equal aliquots. The iROGP in each of these aliquots was separated from the [Cys¹⁵(NEM)]OGP-NH₂ by HPLC using a reverse phase Vydac protein C4 column employing the following acetonitrile gradient run at a flow rate of 1 ml/min: 3 ml 12% acetonitrile; and 30 ml 12-19% acetonitrile.

Separation between two iROGP peaks

[0032] Half ml fractions comprising the main iROGP peak recovered from the C4 column were pooled and partially evaporated to final volume of 400 μ l. The iROGP content of this peak was 0.26 nmol. The filtrate was divided into two equal aliquots. The iROGP in each of these aliquots was subjected to HPLC on a reverse-phase C18 column employing the following acetonitrile gradient run at a flow rate of 1 ml/min: 3 ml 14% acetonitrile and 30 ml 14-19% acetonitrile.

45 Amino acid sequence determination

[0033] Protein in the two iROGP peaks recovered from the C18 column was subjected to automated peptide sequence analysis in an Applied Biosystems 470A sequencer using the program and reagents supplied by the manufacturer. Released amino acid derivatives were identified with the aid of an on-line HPLC system.

50 Results

[0034] Fig. 1 shows that in all three cell systems studied, the total iROGP reached a peak 24 h after starvation and exposure to the chemically defined medium. This time period coincides with the confluency of the cultures. During the next 24 h there was an approximately 50% decrease in the total iROGP. The steady state iROGP showed a more or less linear increase during the whole detection period. These results indicate that stromal cells produce iROGP and the OGPBP. The rate of synthesis of the iROGP and/or the OGPBP decreases when the cultures reach confluency.

[0035] The 24 h culture medium from the MC3T3 E1 cells was subjected to repeated dilution/centrifugation cycles

to remove the non-bound iOGP and other peptides with molecular weight smaller than 3000 (Fig. 2). Only 0.96% of the total iOGP could be discarded in this way. The remaining iOGP was left in the form of OGP-OGPBP complex that was contained in the retentate because of its high molecular weight (Table A). The HPLC separation of [Cys¹⁵(NEM)]OGP-NH₂ resulted in three major light absorption peaks. The greater of these peaks eluted at 19.5-23.5 min retention time, a position similar to that of [Cys¹⁵(NEM)]OGP-NH₂ run separately under the same conditions. This peak did not show OGP immunoreactivity. Both other peaks were only partially separated from each other and showed a high iOGP content (Fig. 1A), 11.5% that of the total iOGP present in the culture medium (Table A). It is likely that most of this decrease in iOGP content resulted from peptidase activity during the displacement reaction, in spite of the presence of peptidase inhibitors. To enhance the separation between these iOGP peaks the respective fractions (retention time 11-12.5 min) were pooled and subjected to a second HPLC step that resulted in two distinct light absorption peaks, both showing high iOGP content (Fig. 1B). These peaks were designated B-1 and B-2.

TABLE A

RECOVERY OF IMMUNOREACTIVE OGP FROM INDIVIDUAL PURIFICATION STEPS		
	iOGP Purification Step (total nmol in preparation)	Percent Recovery
24-h post starvation medium	270.0	100
Initial ultrafiltration	2.6	0.96
Post-displacement ultrafiltration	31.0	11.5
Reverse-phase HPLC	12.6	4.7

[0036] The respective fractions eluted with retention times 23.5-24 min and 25-25.5 min were pooled. Aliquots of the pooled fractions were allocated for testing their proliferative effect in the MC3T3 E1 cell assay. Amino acid sequencing revealed that peak B-1 contained a pentapeptide identical to the C-terminal region (residues 10-14) of OGP. Peak B-2 contained a similar pentapeptide in which Gly¹³ of the OGP was substituted by His (Table B).

TABLE B

AMINO ACID SEQUENCES OF PENTAPEPTIDES	
Peak B-1*	Tyr-Gly-Phe-Gly-Gly
Peak B-2*	Tyr-Gly-Phe-His-Gly

*Defined in Figure 3.

[0037] The proliferative activity of either peak in the MC3T3 E1 cell assay was identical to that of positive sOGP controls with a peak at 10⁻¹³M (Fig 4).

Example 2- Activity of Synthetic Pentapeptides

Peptide synthesis

[0038] Synthetic peptides of this invention were prepared by solid phase method of Merrifield [Merrifield, (1969) Adv. Enzymol. 32: 221] using an Applied Biosystems Model 430A Automated Peptide Synthesizer (Applied Biosystems Inc., Foster City, CA). In the case of peptide free acid the synthesis was carried out on 0.5 mmol t-Boc-Gly-PAM resin (1% cross-linked, 0.61 meq/g). t-Boc-Gly-MBHA resin (1 % cross-linked, 0.66 meq/g) was used for the amidated tetrapeptide. The amino acid derivatives were protected on the α-amino function by t-butyloxycarbonyl (Boc) groups. Protection of the tyrosine side chain was by Z. The amino acid derivatives were coupled via the DCC-mediated preformed symmetrical anhydride method of Hagemair, H. and Frank, H. [Hoppe-Seyler's Z. (1972) Physiol. Chem. 353:1973]. The coupling of each amino acid residue was repeated twice. Deprotection of the blocked amino terminus was by treatment with 25% TFA in DCM. Side chains were deprotected and the peptide cleaved from the resin using

the HF procedure where a mixture of 4 ml anisole and 36 ml liquid HF was used for 75 min at 0°C. The crude synthetic peptides were purified on a Merck-Hitachi 655A-11.HPLC instrument equipped with a Waters μ BondPark™ C18 column (1.9x15.0 cm). The cartridge was pumped with acetonitrile linear gradients containing TFA (Table C) at a flow rate of 6.0 ml/min.


- 5 [0039] The BH-OGP (11-14) (BH = Bolton-Hunter) was prepared by reacting 3-(3-iodo-4-hydroxyphenyl) propionic acid N-hydroxysuccinimidyl ester with purified OGP (11-14) using the method of Michelot *et al* [Michelot R. *et al* (1980) Biochem. Biophys. Res. Comm. 95:491-498].

TABLE C

ACETONITRILE LINEAR GRADIENTS USED FOR HPLC PURIFICATION OF DI-, TRI-, TETRA-, PENTA- AND HEXA-OGP RELATED PEPTIDES			
Peptide	Gradient		
	(% Acetonitrile)	Time (min)	% TFA
OGP (13-14)	0% isocratic	30	.1
OGP (11-12)	0% isocratic	30	.1
OGP (12-14)	0% isocratic	30	.1
OGP (11-13)	0% isocratic	30	.1
OGP (10-12)	0-25	120	.1
OGP (11-14)	0-30	120	.1
BH*-OGP(11-14)	0-25	90	.1
OGP(11-14)NH ₂	0-5	90	.05
OGP(10-13)	0-25	120	.1
OGP(10-14)	0-20	100	.1
OGP(10-14)His ¹³	0-20	100	.1
Ac-Met ^O [OGP(10-14)]	0-20	150	.1
*BH = Bolton-Hunter reagent			

[0040] Table D demonstrates the conversion between the nomenclature used herein and amino acid sequence of the peptides.

TABLE D
AMINO ACID COMPOSITION OF SYNTHETIC
DI-, TRI-, TETRA-, PENTA- AND HEXA- OGP RELATED PEPTIDES

5		
10	OGP (13-14)	Gly-Gly
	OGP (11-12)	Gly-Phe
	OGP (12-14)	Phe-Gly-Gly
15	OGP (11-13)	Gly-Phe-Gly
	OGP (10-12)	Tyr-Gly-Phe
	OGP (11-14)	Gly-Phe-Gly-Gly
20	BH*-OGP(11-14)	 (CH ₂) ₂ CO-Gly-Phe-Gly-Gly
	OGP (11-14) NH ₂	Gly-Phe-Gly-Gly-NH ₂
25	OGP (10-13)	Tyr-Gly-Phe-Gly
	OGP (10-14)	Tyr-Gly-Phe-Gly-Gly
	OGP (10-14) His	Tyr-Gly-Phe-His-Gly
30	Ac-Met ⁰ [OGP(10-14)]	Ac-Met-Tyr-Gly-Phe-Gly-Gly

*BH = Bolton-Hunter reagent

Results

[0041] The proliferative effect of either pentapeptide (OGP (10-14) (formula (e)) or OGP (10-14)His¹³ (formula (a))) in the MC 3T3 E1 and NIH 3T3 cell assays was similar to that of sOGP positive controls. The respective peak activities were at 10⁻¹³ and 10⁻¹¹-10⁻¹⁰M peptide concentration (Fig. 5).

Example 3 - Proliferative effect of hexapeptide

[0042] The hexapeptide of Ac-Met⁰[OGP](10-14)] was prepared for testing the feasibility of using Ac-[³⁵S]Met[OGP(10-14)] for metabolic and binding studies.

Results

[0043] The Ac-Met⁰[OGP](10-14)] stimulated the MC 3T3 E1 and NIH 3T3 cell number in a manner closely similar to that of positive sOGP controls with peaks at 10⁻¹³ and 10⁻¹¹ M peptide concentration, respectively (Fig 6).

Example 4 - Proliferative effect of BH-OGP(11-14), tetra-, tri- and di-peptides

[0044] All the five tetrapeptides had a dose-response effect on the MC 3T3 E1 and NIH 3T3 cell number with peaks at 10⁻¹³ and 10⁻¹¹ M peptide concentration, respectively. The magnitude of the BH-OGP (11-14) peak effect in either cell system was highest and similar to that of the sOGP positive control. The peak activity of the OGP(11-14), OGP(10-

13) and OGP (10-12) showed intermediate values. The OGP (11-14) NH₂ showed the lowest peak effect. OGP (12-14), OGP (11-13), OGP(13-14) and OGP (11-12) did not affect the MC 3T3 E1 and NIH 3T3 cell number (Table E).

TABLE E

PROLIFERATIVE ACTIVITY OF SYNTHETIC DI-, TRI- AND TETRA-OGP RELATED PEPTIDES {Percent Activity of Full Length Peptide [OGP(1-14)]}						
Peptide	MC3T3 E1			NIH 3T3		
	Mean	Range	No. of Expts.	Mean	Range	No. of Expts.
BH*-OGP(11-14)	113	70-137	3	80	63-112	3
OGP(11-14)	66	34-100	8	68	34-100	4
OGP(10-13)	58	42-74	2	89	89	1
OGP(10-12)	41	41	1	57	57	1
OGP(11-14)NH ₂	25	19-31	2	33	31-35	2
OGP(12-14)	0	0	3	0	0	3
OGP(11-13)	0	0	3	0	0	3
OGP(13-14)	0	0	3	0	0	3
OGP(11-12)	0	0	3	0	0	3
*BH - Bolton-Hunter reagent.						

Claims

1. A biochemically pure oligopeptide having stimulatory activity on osteoblastic and/or fibroblastic cells, a molecular weight of from 200 to 1,000, and a formula selected from (a) Tyr-Gly-Phe-His-Gly; (b) Tyr-Gly-Phe-Gly; (c) Ac-Met-Tyr-Gly-Phe-Gly-Gly; and (d) Gly-Phe-Gly-Gly-NH₂.
2. A method of isolating a biochemically pure oligopeptide according to claim 1, part (a), or for isolating a biochemically pure oligopeptide having stimulatory activity on osteoblastic or fibroblastic cells and having the formula (e) Tyr-Gly-Phe-Gly-Gly from a biological sample, which process comprises the steps of:
 - (a) discarding from said biological sample peptides having a molecular weight lower than 3000; to leave a medium.
 - (b) incubating the medium obtained in step (a) with a polypeptide that binds to an osteogenic growth polypeptide binding protein/s and does not bind to an antibody directed against osteogenic growth polypeptide in the presence of protease inhibitors to compete said osteogenic growth polypeptide out from its complex with said osteogenic growth polypeptide binding protein/s; and
 - (c) separating the immunoreactive osteogenic growth peptide from the reaction medium obtained in step (b) by chromatographic methods.
3. A method according to claim 2 wherein, in step (a), the peptides having molecular weights lower than 3000 are discarded by ultra-filtration with a cut-off of 3000MW.
4. A method according to claim 2 or claim 3 wherein said protease inhibitors are E-64, Leupeptin or PMSF or mixtures thereof.
5. A method according to any one of claims 2 to 4 wherein, in step (c), the immunoreactive osteogenic growth peptide is separated by HPLC.
6. A pharmaceutical composition comprising an oligopeptide according to claim 1, or having the formula (f) Gly-Phe-Gly-Gly; and a pharmaceutically acceptable carrier.

7. A method of preparing a pharmaceutical composition comprising formulating an oligopeptide or modified oligopeptide as defined in claim 6 with a pharmaceutically acceptable carrier.
8. An oligopeptide according to claim 1, or having the formula (f) Gly-Phe-Gly-Gly for use in a method of treatment of the human or animal body by surgery or therapy or in diagnosis.
9. An oligopeptide according to claim 8 wherein the method of treatment is for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, and other conditions requiring enhanced bone formation.
10. Use of an oligopeptide according to claim 1, or having the formula (e) Tyr-Gly-Phe-Gly-Gly, or (f) Gly-Phe-Gly-Gly in the manufacture of a medicament for the stimulation of formation of osteoblastic or fibroblastic cells, enhanced bone formation in osteopenic pathological conditions, fracture repair, healing of wounds, intraosseous implants and bone supplementation, and other conditions requiring enhanced bone formation.

Patentansprüche

1. Biochemisch reines Oligopeptid mit stimulierender Wirkung auf osteoblastische und/oder fibroblastische Zellen, einem Molekulargewicht von 200 bis 1.000 und einer Formel, gewählt aus (a) Tyr-Gly-Phe-His-Gly; (b) Tyr-Gly-Phe-Gly; (c) Ac-Met-Tyr-Gly-Phe-Gly-Gly; und (d) Gly-Phe-Gly-Gly-NH₂.
2. Verfahren zur Isolierung eines biochemisch reinen Oligopeptids nach Anspruch 1, Teil (a), oder zur Isolierung eines biochemisch reinen Oligopeptids mit stimulierender Wirkung auf osteoblastische oder fibroblastische Zellen und mit der Formel (e) Tyr-Gly-Phe-Gly-Gly aus einer biologischen Probe, welches Verfahren die folgenden Schritte umfaßt:
 - (a) Entfernen aus der biologischen Probe der Peptide mit einem Molekulargewicht von unter 3.000; zur Zurückbehaltung eines Mediums.
 - (b) Inkubieren des in Schritt (a) erhaltenen Mediums mit einem Polypeptid, das an ein osteogenes Wachstums-Polypeptid-bindendes Protein/e bindet und nicht an einen gegen das osteogene Wachstums-Polypeptid gerichteten Antikörper bindet, in Gegenwart eines Protease-Inhibitors, um das osteogene Wachstums-Polypeptid aus seinem Komplex mit dem osteogenen Wachstums-Polypeptidbindenden Protein/en herauszukurrieren; und
 - (c) Abtrennen des immunreaktiven osteogenen Wachstumspeptids aus dem in Schritt (b) erhaltenen Reaktionsmedium mittels chromatographischer Methoden.
3. Verfahren nach Anspruch 2, wobei, in Schritt (a), die Peptide mit einem Molekulargewicht von unter 3.000 mittels Ultrafiltration bei einem Rückhaltevermögen von 3.000 MG entfernt werden.
4. Verfahren nach Anspruch 2 oder Anspruch 3, wobei die Protease-Inhibitoren E-64, Leupeptid oder PMSF oder deren Gemische sind.
5. Verfahren nach einem der Ansprüche 2 bis 4, wobei, in Schritt (c), das immunreaktive osteogene Wachstumspeptid mittels HPLC abgetrennt wird.
6. Pharmazeutische Zusammensetzung, umfassend ein Oligopeptid nach Anspruch 1, oder mit der Formel (f) Gly-Phe-Gly-Gly; und einen pharmazeutisch geeigneten Träger.
7. Verfahren zur Herstellung einer pharmazeutischen Zusammensetzung, umfassend das Formulieren eines Oligopeptids oder modifizierten Oligopeptids, wie in Anspruch 6 definiert, mit einem pharmazeutisch geeigneten Träger.
8. Oligopeptid nach Anspruch 1, oder mit der Formel (f) Gly-Phe-Gly-Gly, zur Verwendung bei einem Verfahren zur Behandlung des menschlichen oder tierischen Körpers durch Chirurgie oder Therapie, oder in der Diagnostik.
9. Oligopeptid nach Anspruch 8, wobei das Behandlungsverfahren zur Stimulierung der Bildung osteoblastischer

oder fibroblastischer Zellen, einer vermehrten Knochenbildung bei osteopenisch pathologischen Zuständen, der Bruchheilung, der Wundheilung, von intraossalen Implantaten und der Knochensupplementation, und anderer Zustände, die eine vermehrte Knochenbildung erfordern, dient.

- 5 10. Verwendung eines Oligopeptids nach Anspruch 1, oder mit der Formel (e) Tyr-Gly-Phe-Gly-Gly, oder (f) Gly-Phe-Gly-Gly, bei der Herstellung eines Medikaments zur Stimulierung der Bildung osteoblastischer oder fibroblastischer Zellen, einer vermehrten Knochenbildung bei osteopenisch pathologischen Zuständen, der Bruchheilung, der Wundheilung, von intraossalen Implantaten und der Knochensupplementation, und anderer Zustände, die eine vermehrte Knochenbildung erfordern, dient.

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Revendications

1. Oligopeptide biochimiquement pur ayant une activité stimulatrice sur les cellules ostéoblastiques et/ou fibroblastiques, une masse moléculaire allant de 200 à 1000, et une formule choisie parmi (a) Tyr-Gly-Phe-His-Gly ; (b) Tyr-Gly-Phe-Gly ; (c) Ac-Met-Tyr-Gly-Phe-Gly-Gly et (d) Gly-Phe-Gly-Gly-NH₂.
- 15 2. Méthode d'isolement d'un oligopeptide biochimiquement pur selon la revendication 1, partie (a), ou pour l'isolement d'un oligopeptide biochimiquement pur ayant une activité stimulatrice sur les cellules ostéoblastiques ou fibroblastiques et ayant la formule (e) Tyr-Gly-Phe-Gly-Gly, à partir d'un échantillon biologique, ce procédé comprenant les étapes consistant :
- 20 (a) à éliminer dudit échantillon biologique les peptides ayant une masse moléculaire inférieure à 3000, pour obtenir un milieu ;
- (b) à incuber le milieu obtenu dans l'étape (a) avec un polypeptide qui se lie à une ou des protéines de liaison au polypeptide de croissance ostéogénique et ne se lie pas à un anticorps dirigé contre un polypeptide de croissance ostéogénique en présence d'inhibiteurs de protéase pour libérer par compétition ledit polypeptide de croissance ostéogénique de son complexe avec ladite ou lesdites protéines de liaison au polypeptide de croissance ostéogénique ; et
- 25 (c) à séparer le peptide de croissance ostéogénique immunoréactif du milieu de réaction obtenu dans l'étape (b) par des méthodes chromatographiques.
3. Méthode selon la revendication 2, dans laquelle, dans l'étape (a), les peptides ayant des masses moléculaires inférieures à 3000 sont éliminés par ultrafiltration avec un seuil de coupure de 3000MM.
- 35 4. Méthode selon la revendication 2 ou la revendication 3, dans laquelle lesdits inhibiteurs de protéase sont l'E-64, la Leupeptine ou le PMSF ou des mélanges de ceux-ci.
5. Méthode selon l'une quelconque des revendications 2 à 4, dans laquelle, dans l'étape (c), le peptide de croissance ostéogénique immunoréactif est séparé par HPLC.
- 40 6. Composition pharmaceutique comprenant un oligopeptide selon la revendication 1, ou ayant la formule (f) Gly-Phe-Gly-Gly ; et un véhicule pharmaceutiquement acceptable.
7. Méthode de préparation d'une composition pharmaceutique comprenant la formulation d'un oligopeptide ou d'un oligopeptide modifié comme défini dans la revendication 6 avec un véhicule pharmaceutiquement acceptable.
- 45 8. Oligopeptide selon la revendication 1, ou ayant la formule (f) Gly-Phe-Gly-Gly, destiné à être utilisé dans une méthode de traitement de l'organisme humain ou animal par chirurgie ou thérapie ou en diagnostic.
9. Oligopeptide selon la revendication 8, dans lequel la méthode de traitement est destinée à la stimulation de la formation de cellules ostéoblastiques ou fibroblastiques, la formation osseuse activée dans des états ostéopéniques pathologiques, la réparation de fracture, la cicatrisation de plaie, les implants intraosseux et la supplémentation osseuse, et d'autres états nécessitant une formation osseuse activée.
- 50 10. Utilisation d'un oligopeptide selon la revendication 1, ou ayant la formule (e) Tyr-Gly-Phe-Gly-Gly ou (f) Gly-Phe-Gly-Gly dans la fabrication d'un médicament pour la stimulation de la formation de cellules ostéoblastiques ou fibroblastiques, la formation osseuse activée dans des états ostéopéniques pathologiques, la réparation de fracture, la cicatrisation de plaie, les implants intraosseux et la supplémentation osseuse, et d'autres états nécessitant
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une formation osseuse activée.

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Fig.1.

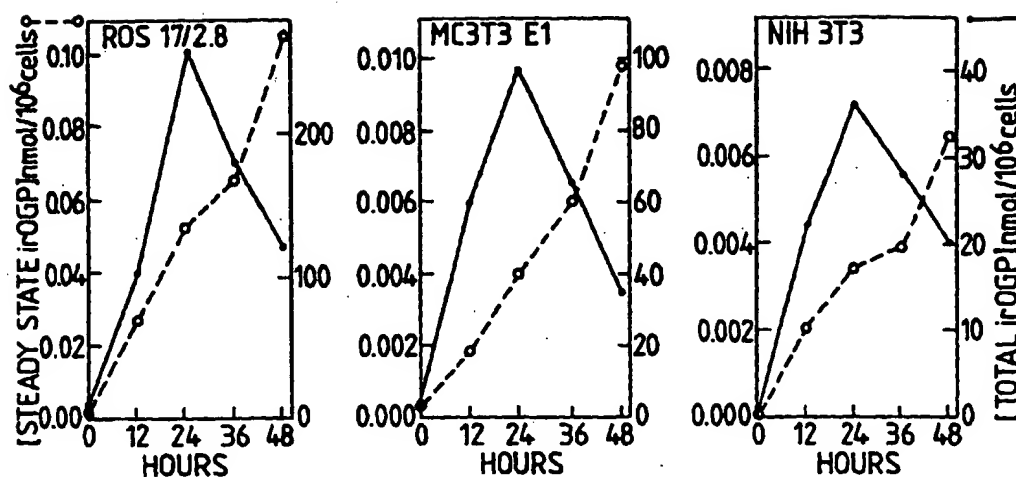


Fig.2.

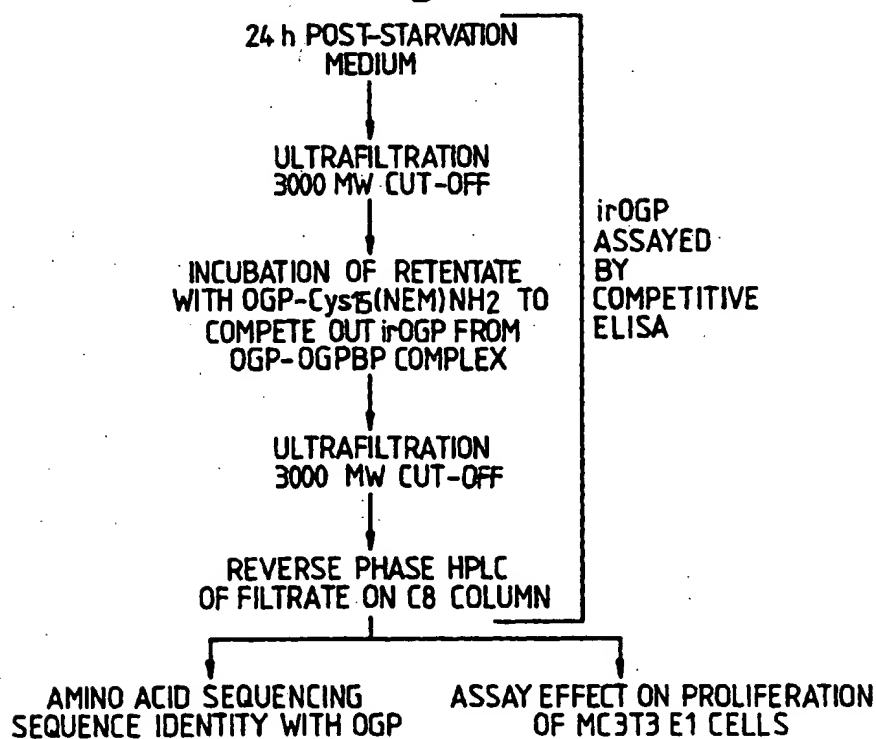


Fig. 3.

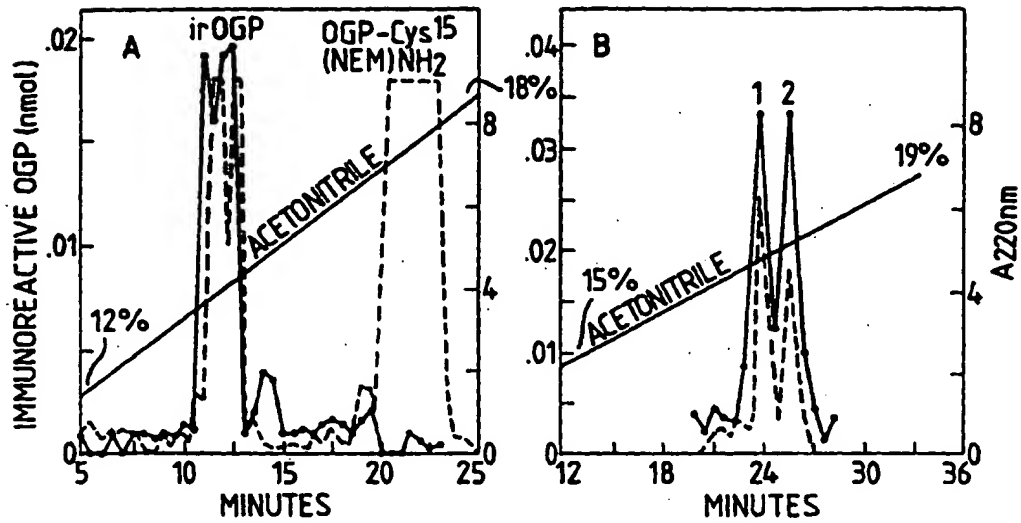


Fig. 4.

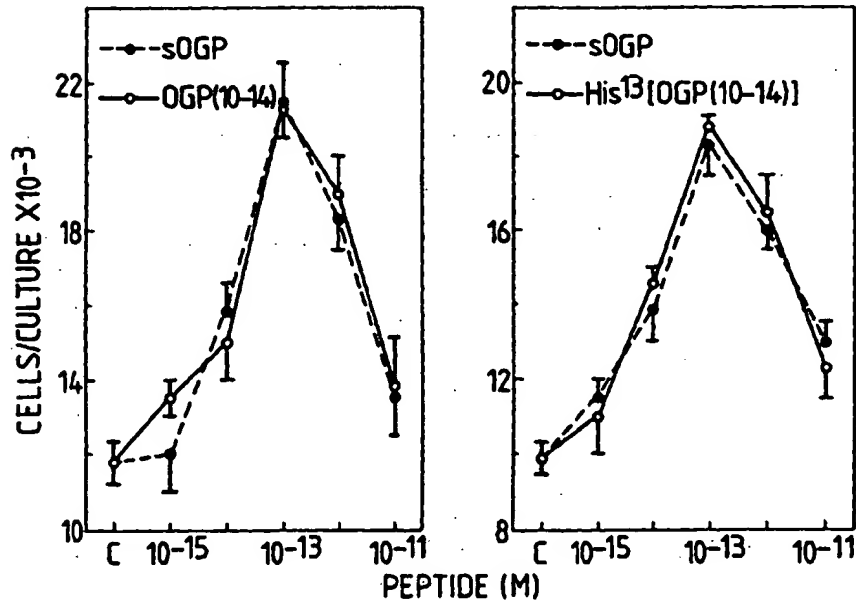


Fig. 5.

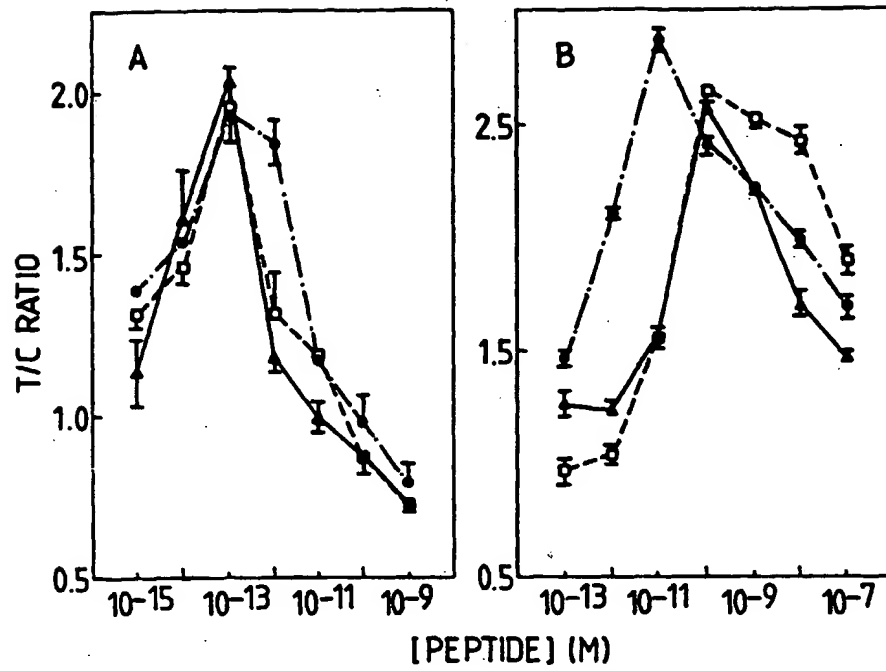


Fig. 6.

